

# Accelerating the Drug Optimization Process: Identification, Structure Elucidation, and Quantification of in Vivo Metabolites Using Stable Isotopes with LC/MS<sup>n</sup> and the Chemiluminescent Nitrogen Detector

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Most preclinical leads exhibit poor ADME/PK properties and require optimizing to increase the likelihood of becoming successful pharmaceuticals. As a means of accelerating the evaluation of these leads in vivo, we assessed the use of LC/MS with the chemiluminescent-nitrogen detector (CLND) and a stable isotope to identify and quantify in vivo metabolites and to measure excretion. A <sup>14</sup>C-labeled preclinical lead that also contained two chlorine atoms was administered orally to rats, and samples of bile, urine, and plasma were collected and analyzed by LC with radiodetection and by LC/MS-CLND with the chlorine atoms used as tracers. Both methods identified seven metabolites in bile and two metabolites in urine. The amount and abundance of each metabolite was measured, and the results were equivalent for the two methods. Material balance was measured by liquid scintillation counting of the starting samples, by LC/radiodetection, and by LC/MS-CLND. All three methods yielded the same results and showed that the primary route of clearance was metabolism followed by immediate excretion. This study demonstrates that LC/MS-CLND with a stable isotope is a method that can efficiently track and accurately quantify metabolites, making it possible to rapidly study ADME/PK in vivo without radiolabeling.

Advances over the past decade in the areas of computational chemistry, split-and-mix and parallel chemical synthesis, and high-throughput screening have revolutionized the drug discovery process by greatly improving the efficiency of generating biologically active "hits". The increase in the rate of identifying hits has in turn put more pressure on early development by providing more compounds to be evaluated as potential drug candidates.<sup>1,2</sup> The primary role of early development is to evaluate the in vivo properties of preclinical leads and to identify problems that may result in a lack of efficacy, in toxicity, or both.<sup>3</sup> These problems

stem from properties related to absorption, distribution, metabolism, excretion, and plasma half-life (ADME/PK) and must be overcome if the drug discovery process is to result in a clinical success.

Most early development groups have responded to the increasing numbers of compounds to evaluate by employing processes similar to those used in the hit generation process, namely, to exploit screens that use less compound, are faster, and can provide critical information for less money. It is now common for ADME/PK screens to be incorporated early into compound optimization strategies so that only compounds with favorable activity, selectivity, and ADME/PK characteristics are studied more thoroughly in vivo.<sup>4</sup> For example, in vitro monolayer permeability assays for absorption, in vitro parent stability assays for metabolism, and in vivo cassette dosing for plasma half-life are being performed in parallel with primary in vitro screens for bioactivity and selectivity. Although in vitro ADME screens can be predictive of in vivo properties, they do not always correlate with the in vivo behavior and, therefore, should not necessarily be considered surrogates for in vivo ADME/PK studies unless an in vitro-in vivo correlation has been established.

Currently, the preferred method for following the fate of a drug in vivo is to administer a radiolabeled form of the compound, typically one that incorporates <sup>14</sup>C into the structural core (see ref 5 for a review). A radiolabel is an ideal tracer because of the ease with which it can be detected and quantified. Hence, the incorporation of a radioisotope into the core of the molecule permits the detection and quantification of biotransformation products (metabolites) and provides a reliable measure of material balance of the administered compound. Radioactivity is the gold standard for tracing and measuring the ADME properties of a drug candidate, but there are many drawbacks to its use with compounds at the discovery-development interface. For example, the synthesis and purification of the radiolabeled compound, the performing of radioactive animal studies, the processing and storing of radioactive samples, and the disposal of samples that are both radioactive and biohazardous make radioactive studies time-consuming, difficult, dangerous, and expensive. In addition,

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radioactive studies require specialized machines for analysis such as a tissue oxidizer, whole body autoradiography equipment, an HPLC radioactivity detector, and typically, a scintillation counter. Therefore, the costs and lead time required to perform radioactive studies make them impractical and undesirable early in the drug discovery and optimization processes where they could make the greatest impact.

LC/MS is used extensively to identify drug metabolites, but it is not used quantitatively unless authentic standards of the metabolites are available (see ref 6 for review). It has been shown previously that the CLND is an excellent method for quantification of compounds containing nitrogen using an external standard.<sup>7</sup> The only requirement for quantification, because of the equimolar response of the detector, is knowledge of the number of nitrogen atoms in the analyte. We also demonstrated the utility of using CLND in conjunction with LC/MS for simultaneous compound identification and quantification. Lewis et al. recently showed that the sole exception to equimolar response of the CLND arises from chemical substructures containing adjacent nitrogen atoms, such as azo groups, presumably because the adjacent nitrogen atoms are released as  $N_2$  gas upon incineration.<sup>8</sup> However, the authors went on to show that this deviation from equimolarity is predictable and can be corrected for in a quantitative manner.

In this study, we introduce and validate a novel way of using LC/MS-CLND to study ADME properties and to measure material balance in vivo. Using a compound containing a radioisotope ( $^{14}C$ ) and a stable isotope ( $^{13}C$ ), a preclinical study was analyzed by both standard radiometric methods and by LC/MS-CLND to identify and quantify metabolites and to determine the major routes of clearance of the compound. The goal of this analysis was to show that the combination of inexpensive and easy to handle stable isotopes with LC/MS-CLND is an alternative to the use of a radiolabel. The use of stable isotopes with MS analysis provides a specific tracer for identification in complex matrixes, and the CLND permits accurate quantification without authentic standards. The results will demonstrate that this methodology provides a means to keep pace with the study of analogues in the lead optimization process and to understand the in vivo behavior of compounds significantly faster and in a more cost-effective manner.

## EXPERIMENTAL SECTION

**Reagents.** CHIR 99021 was synthesized at Chiron Corporation (Emeryville, CA). [ $^{14}C$ ]CHIR 99021 was synthesized at Wizard Laboratories (West Sacramento, CA), and both were supplied as dry powders. Reference standard grade diphenhydramine was purchased from Sigma Chemical Co. (St. Louis, MO). HPLC grade methanol (MeOH) and 2-propanol (IPA) were purchased from Fisher Scientific (Pittsburgh, PA). Water was purified through a Milli-Q plus TOC water purification system (Millipore Corporation, Bedford, MA). Sequencing grade trifluoroacetic acid (TFA) was purchased from Pierce Chemical (Rockford, IL).

**Instrumentation.** Chromatography was performed on a Shimadzu VP Series HPLC system (Columbia, MD) equipped with a microflowcell, a 25- $\mu$ L static mixer (Analytical Scientific Instruments, El Sobrante, CA) and  $\pm 0.005$  in. internal diameter (I.D.) PEEK tubing throughout. Owing to the difficulty in rendering an HPLC system "nitrogen free," this HPLC is dedicated for quantitation work requiring the CLND. Following UV detection, the eluate was either split  $\sim 9:1$  by a microvolume Y-connector (Valco Instrument Co., Inc., Houston, TX) between a CLND (model 8060, Antek Instruments, Inc., Houston, TX) and an L-10 ion trap mass spectrometer (Finnigan, San Jose, CA), respectively, or directly into a RAM mass spectrometer (Finnigan, San Jose, CA). The L-10 is equipped with a 200- $\mu$ L liquid trap.

**Animal Studies.** Male Sprague-Dawley rats (250–300 g) were cannulated in the bile duct, allowed to recover for one week, then cannulated in both the jugular and femoral veins and allowed to recover for 2 days. Animals were provided rat chow and water ad libitum and maintained on a 12 h light/dark schedule during recovery periods. Dosing solutions were prepared by dissolving CHIR 99021 in 10 mM citrate/1.4% dimethylacetamide at 2.5 mg/mL and in 10 mM citrate/2.8% (v/v) dimethylacetamide at 3 mg/mL for intravenous (IV) and per oral (PO) administration, respectively. The appropriate amount of [ $^{14}C$ ]CHIR 99021 was mixed with unlabeled CHIR 99021 in each dosing solution such that  $\sim 5$   $\mu$ Ci was delivered to each rat. Following fasting for 1–3 h, animals were dosed by IV bolus at 5 mg/kg or by oral gavage at 30 mg/kg. Bile was continuously collected for 8 h in 2 h increments and urine was collected after 4 h, 8 h, and 24 h. Samples were stored at  $-20$  °C until analysis. A total of 3 rats/route of administration were used. Rats were euthanized by lethal injection following the last sample collection.

**Analysis of Samples.** Samples at each collection period were pooled. Bile and urine samples were centrifuged at 14000g for 5 min. Plasma samples were precipitated with 3 $\times$  volume of acetonitrile, dried under vacuum, then reconstituted with 10% MeOH, 0.1% TFA in 1/5 of the initial plasma volume. For LC/UV-CLND-MS analysis, 2.5, 5, and 10  $\mu$ L each of bile, urine, and plasma sample, respectively, was injected onto a BetaBasic  $C_{18}$  column, 1  $\times$  150 mm, 5- $\mu$ m particle size (Thermo Hypersil-Keystone, Bellefonte, PA), and the column was eluted at 100  $\mu$ L/min with a linear gradient from 20 to 40% solvent B in 30 min. Solvent A was 0.1% (v/v) TFA in water and solvent B was 0.08% (v/v) TFA in 75% (v/v) MeOH, 25% (v/v) IPA. For urine samples, the column was washed with 10% solvent B for 10 min before starting the gradient to remove uric acid and other unbound nitrogen-containing compounds that would cause interference. The CLND settings for inlet oxygen, makeup argon, ozone oxygen, and high voltage were 225, 200, and 40 mL/min, and 750 V, respectively. MS data were collected in positive mode using the electrospray interface equipped with a metal capillary. The source voltage was set to 4.5 kV; the sheath gas, to 60; and the heated desolvation capillary was held at 260 °C. Spectra were acquired with an automated MS<sup>2</sup> method using a relative collision energy for CID of 45. This method incorporated four scan events—one MS scan, one MS<sup>2</sup> scan, and two MS<sup>3</sup> scans—and utilized dynamic exclusion to prevent isolation and CID of only the most abundant ion and its related isotope ions. Data-dependent MS/MS was performed on the most abundant ion in the parent ion scan with

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an intensity greater than  $1 \times 10^6$  counts, then data-dependent MS<sup>3</sup> was performed on the most and second-most abundant ions in the MS/MS scan. The scan range for the parent scan was 300–800 *m/z*, and each scan consisted of 3 microscans with an auto gate control (AGC) target of 50 ms. The global isolation width for CID was set to 6 amu, and a parent ion default charge state was set to +1 to calculate the scan range for the product ion scans. Each MS<sup>3</sup> scan consisted of 5 microscans with an AGC target of 200 ms. The global dynamic exclusion settings for exclusion mass, repeat count, repeat duration, and exclusion duration were 6 amu, 5 scans, 1 min, and 1 min, respectively.

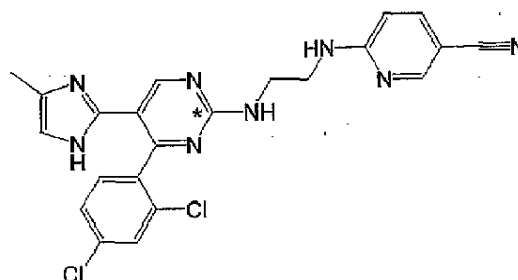
Total radioactivity of each sample was measured by liquid scintillation counting (LSC). For RP-HPLC with radiodetection, 25  $\mu$ L of each bile and urine sample was injected onto a BetaBasic C<sub>18</sub> column, 2  $\times$  150 mm, 5- $\mu$ m particle size, and the column was eluted at 400  $\mu$ L/min with a linear gradient from 20 to 40% solvent B in 30 min using the solvents described above. The eluate was directed to the  $\beta$ -RAM where it was mixed with IN-FLOW ES LSC cocktail (IN/US) at a scintillant-to-eluate ratio of 4:1, resulting in a flow rate of 2 mL/min in the flow cell and residence time of  $\sim$ 7 s. For the analogue output from the  $\beta$ -RAM, the time per point was set to 5 s; smoothing, to 3 points; and the counts at full scale, to 50. The sensitivity and accuracy of the online detection was corroborated by collecting fractions over an entire chromatogram and counting all fractions off-line by LSC.

For quantification of metabolites by the CLND and radioactivity chromatograms, integration parameters were optimized for each detector, and the same peak integration parameters were used for all samples. Calibration curves were fit using linear regression with  $1/x^2$  weighting. The CLND and the  $\beta$ -RAM were calibrated using diphenhydramine and the IV dosing solution, respectively.

## RESULTS AND DISCUSSION

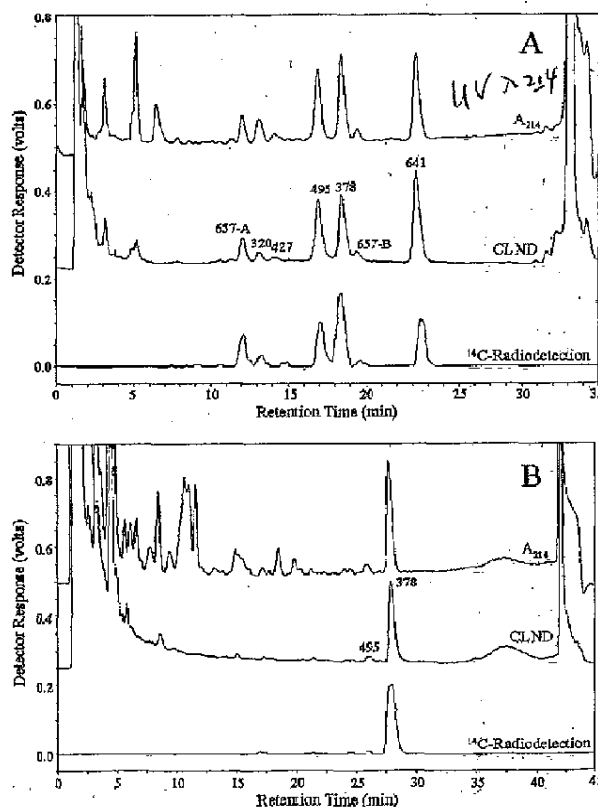
The purposes of this study were to follow the *in vivo* fate of a preclinical lead via radiolabel and to explore using LC/CLND-MS as an alternative means to the same end. We reasoned that if we could track and quantify the parent compound and its metabolites *in vivo* using LC/CLND-MS, we would be able to provide the same distribution, metabolism, excretion, and material balance data to preclinical studies without employing radioactivity. This, in turn, would enable analogues to be assessed *in vivo* more rapidly to determine their ADME properties, which is particularly important when *in vitro* assays are not a surrogate for *in vivo* behavior, or when an *in vitro*–*in vivo* correlation has yet to be established.

The compound analyzed in this study was derived from an optimization series of which all the members that exhibited efficacy *in vivo* following PO administration had plasma half-lives of <60 min and clearance rates faster than liver and kidney blood-flow combined. Although rapid metabolism was suspected as the reason for the short half-lives, no correlation existed between plasma half-lives and metabolism *in vitro* using either microsomes or primary hepatocytes (data not shown). Thus, to evaluate the reason for the short plasma half-life and the rapid clearance, the most efficacious compound from this optimization series, CHIR 99021, was radiolabeled with <sup>14</sup>C (Figure 1) and administered orally to rats in order to determine its metabolism and excretion properties *in vivo*.

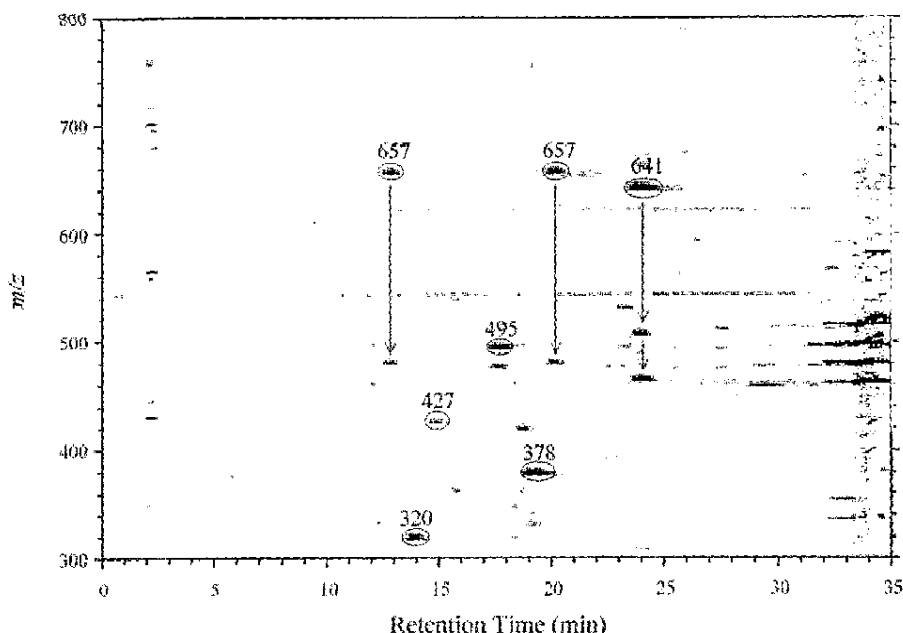


**Figure 1.** Chemical structure of CHIR 99021. An asterisk (\*) denotes the position of the <sup>14</sup>C.

Similar to quantification of compounds by an on-line radioactivity detector, quantification by CLND requires that the compounds to be individually quantified be separated from one another. CLND also requires separation of the analyte from other nitrogen-containing components in the sample or matrix. To achieve the necessary resolution for the metabolites in our samples, the slope of the gradient was reduced to 0.67% B/min over the region where these compounds eluted. All of the metabolites were well-resolved by the chromatographic conditions used (Figure 2) and were observed to be stable. There was no significant interference from



**Figure 2.** Chromatograms from RP-HPLC analysis of 4–6 h bile and 4–8 h urine. The UV and CLND traces are from the separation on the 1-mm column and the radiodetector trace was overlaid from the separation on the 2-mm column. Peaks corresponding to metabolites are labeled by the  $MH^+$  ion detected from the MS analysis. A and B denote the two observed species of 657  $MH^+$  ion. Note that the ratio of the peak heights between the CLND and radiodetector for each metabolite varies depending on the number of nitrogen atoms in the metabolite.



**Figure 3.** MS analysis of 4–6 h bile following RP-HPLC. The entire MS file is presented as a density plot in which intensity correlates to darkness of the spot. Metabolites were identified by the isotopic pattern that is characteristic of chlorine atoms and are circled. Arrows show ions formed by in-source fragmentation of the parent ion. The large bands of ions eluting at 2 and 34 min are the flow-through and column wash, respectively.

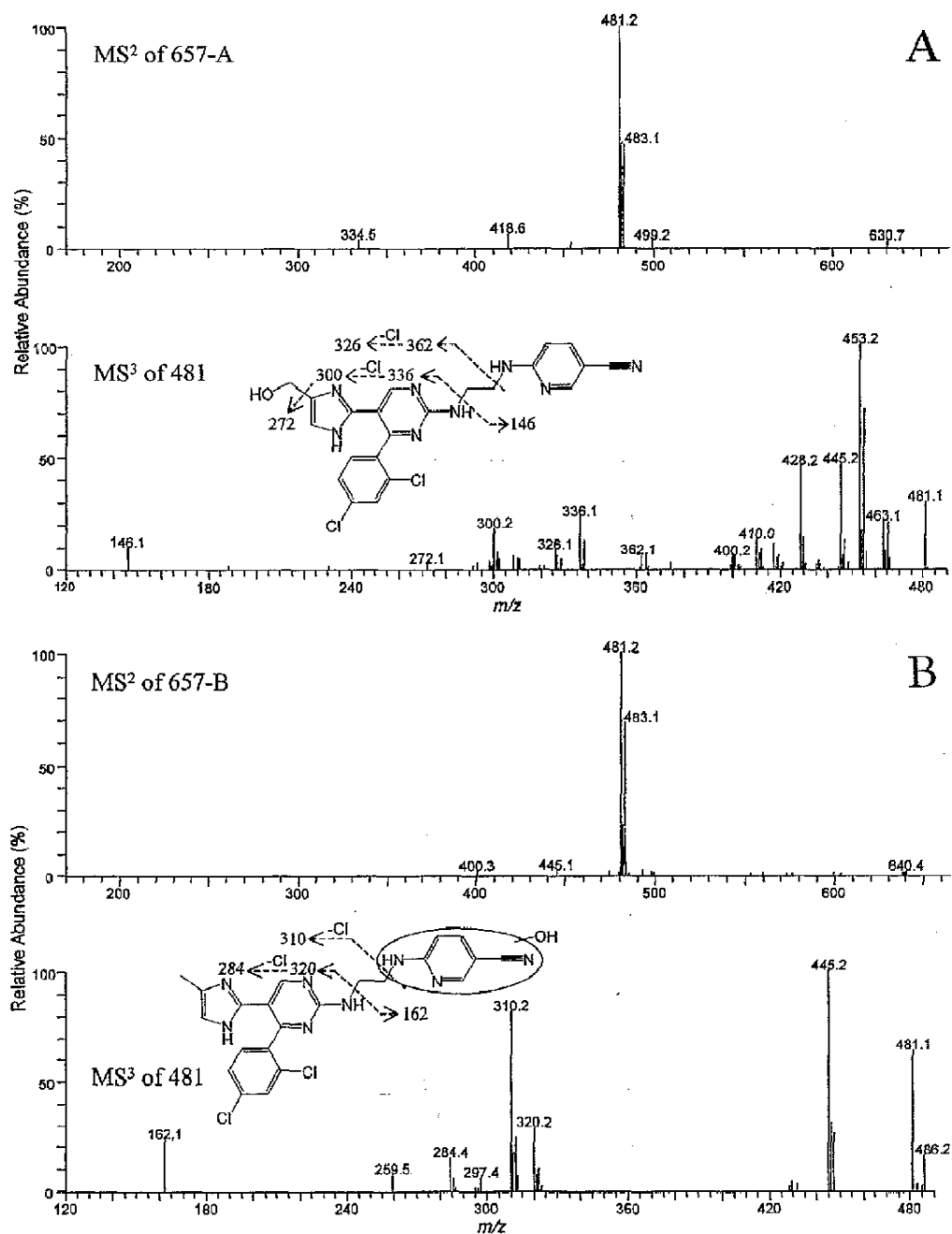
nitrogen-containing compounds in any of the matrixes we examined, including those that were precipitated with acetonitrile (plasma, liver, kidney, and muscle). The lack of interference from nitrogen-containing compounds in the matrixes was unexpected. The limit of detection by CLND was  $\sim 25$  pmol of nitrogen injected, which translated to  $\sim 3$ – $5$  pmol of compound.

CHIR 99021 contains two chlorine atoms, and the distinctive isotope pattern resulting from the presence of these atoms, an isotopic ion 2 amu heavier than and 65% of the intensity of the monoisotopic ion, was used to identify it and related compounds by MS. To make the identification process thorough and unbiased, each MS file was represented as a density plot for identification of metabolites on the basis of this pattern. Using this technique, metabolites 657 and 641 were identified (Figure 3). This number of metabolites was corroborated by the  $^{14}\text{C}$ -radiodetector trace (Figure 2A), where seven peaks were evident, each with the same retention time as a metabolite peak in the CLND trace as identified by MS. For the 641  $\text{MH}^+$  and 657  $\text{MH}^+$  ions that represented *N*-glucuronidated (+176 amu) and *O*-glucuronidated (+192 amu) metabolites, respectively, some in-source fragmentation was observed (Figure 3). In urine, two metabolites were identified (Figure 2B).

To elucidate the structure of the metabolites from a single chromatographic analysis, a data-dependent  $\text{MS}^2$  method that provided  $\text{MS}^2$  and  $\text{MS}^3$  data was used. To retain the chlorine isotope pattern in the fragment ions, all precursor ions were isolated at a width of  $\pm 3$  amu. Two  $\text{MS}^2$  scan events were utilized for further structural information on the two most intense ions in the  $\text{MS}^2$  scan. To ensure that the two  $\text{MS}^2$  scans were of different structures and not isotope ions of the same structure, a dynamic exclusion width of  $\pm 3$  amu forced fragmentation of ions that were  $> 3$  amu apart. An example of the utility of this method is shown

in Figure 4. The  $\text{MS}^2$  spectra of the two 657  $\text{MH}^+$  metabolites show that fragmentation of both precursor ions principally yielded the neutral loss of glucuronic acid (176 amu) and a fragment ion representing a hydroxylated parent compound ( $465 + 16$  amu). The most useful information was derived from the  $\text{MS}^2$  fragmentation patterns. Comparing these two  $\text{MS}^2$  spectra, the location of hydroxylation on metabolite 657-A was narrowed by the presence of the 300-fragment ion, which was 16 amu larger than the corresponding 284-fragment ion from 657-B. The presence of the 272-fragment ion suggested that the site of hydroxylation was the methyl group on the imidazole. For metabolite 657-B, the location of hydroxylation was narrowed by the presence of the 162-fragment ion, as compared to the corresponding 146-fragment ion from 657-A, and the 310-fragment ion, which eliminated the ethyl linker. These data showed that the metabolites were conjugated at different locations on the molecule: 657-A on the methimidazole and 657-B on the aminocyanopyridine. The proposed structure of each metabolite as determined by this method is shown in Table 1.

The metabolites identified by MS were quantified using the  $^{14}\text{C}$ -radiodetector traces and the CLND traces in Figure 2, and the results are summarized in Table 1. For quantification via radioactivity, amounts were determined relative to the dosing solution. For the CLND, amounts were determined relative to an external standard after correcting for the number of nitrogen atoms in each metabolite. The precision and accuracy of quantification of both detectors were  $\pm 3$  and  $\pm 15\%$ , respectively. Within expected error, the amounts of all metabolites excreted into the bile and into the urine at these collection periods were equivalent by the two methods. This equivalency was further illustrated by the similarity of the relative abundances measured for each metabolite. Since both the  $^{14}\text{C}$ -radiodetector and the CLND allow



**Figure 4.** On-line MS<sup>n</sup> analysis of the 657 MH<sup>+</sup> ions. The MS<sup>2</sup> and MS<sup>3</sup> spectra of metabolite 657-A and of 657-B are in panels A and B, respectively. Each spectrum shown is the average of 3 spectra. The proposed structure and fragmentation patterns of hydroxylated product ions (481  $m/z$ ) are shown.

for the quantification of individual metabolites, the excretion profile of the major metabolite, 378 MH<sup>+</sup>, was examined. As shown in Figure 5, both detection methods produced the same results, with excretion into bile reaching a maximum by 6 h and the majority of the excretion into urine occurring after 8 h. In addition to the similar profiles, the absolute amounts of this metabolite measured in each sample were equivalent for these two methods. These data show that the CLND can be used to accurately quantify metabolites in complex matrixes.

To evaluate use of the CLND for measuring material balance of the administered compound, three methods for measuring cumulative excretion into bile and urine were compared: summing radioactivity by liquid scintillation counting, summing radioactivity by LC with radiodetection, and summing amounts by LC/MS-CLND. The precision and accuracy of counting by liquid scintillation were 1 and 5%, respectively. As summarized in Table 2, the three methods yielded the same results, within experimental error. The percentages of the dose excreted into the bile and the urine

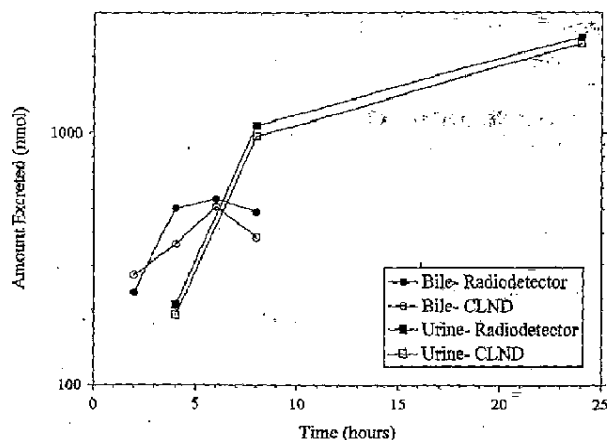
**Table 1. Quantitation of Metabolites in 4–6 h Bile and 4–8 h Urine**

Metabolite (MH <sup>+</sup> )	Structure <sup>a</sup>	Bile				Urine			
		<sup>14</sup> C-Radiodetection		CLND		<sup>14</sup> C-Radiodetection		CLND	
		Amount (nmol) <sup>b</sup>	Percent Abundance	Amount (nmol) <sup>b</sup>	Percent Abundance	Amount (nmol) <sup>b</sup>	Percent Abundance	Amount (nmol) <sup>b</sup>	Percent Abundance
465 (parent)		<LOD	-	<LOD	-	<LOD	-	<LOD	-
657-A		200	13	110	7	<LOD	-	<LOD	-
320		68	5	79	5	<LOD	-	<LOD	-
427		24	2	36	2	<LOD	-	<LOD	-
495		300	20	370	24	<LOD	-	<LOD	-
378		540	36	510	33	40	4	60	6
657-B		35	2	71	5	1070	96	970	94
641		320	22	360	23	<LOD	-	<LOD	-

<sup>a</sup> Structures of metabolites were proposed on the basis of MS<sup>2</sup> and MS<sup>3</sup> data. <sup>b</sup> Amounts were determined by integrating the peak of each metabolite, calculating the amount injected on the column relative to a calibration standard, then correcting for the total volume of sample. Values are average per animal. <LOD: Below the limit of detection, which was ~5 pmol of compound for both detectors.

were ~35 and 25%, respectively; ~60% cumulatively. In both bile and urine, the parent compound was not detected (Figure 2 and Table 1), suggesting that it was not excreted unchanged. In plasma samples taken during this study, only parent compound was detected (data not shown), suggesting that the metabolites were cleared immediately. In addition, the proportion of the dose cumulatively excreted into bile and urine following IV administra-

tion was ~85% (data not shown) suggesting that the compound was not completely absorbed in the gut. These results show that CHIR 99021 is rapidly and extensively metabolized in vivo and that the major route of clearance is via metabolism followed by immediate excretion of the metabolites into bile and urine. Furthermore, the equivalent results obtained via radiodetection and LC/CLND show that the latter method accurately measured material balance in vivo and, therefore, can expedite in vivo studies by obviating the current need for a radiolabel.

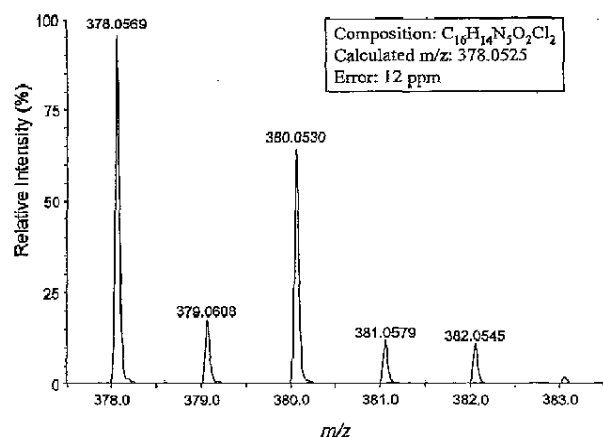


**Figure 5.** Profile of 378 MH<sup>+</sup> metabolite excretion into bile and urine, as measured by radiodetection and by CLND.

**Table 2. Material Balance of CHIR 99021 Measured by Three Methods**

sample	percent of dose		
	LSC <sup>a</sup>	LC/radiodetection <sup>b</sup>	LC/CLND <sup>c</sup>
bile	38	33	35
urine	24	24	22
cumulative excretion	62	57	57

<sup>a</sup> The cumulative cpm in each collection period was measured, summed, then divided by the total cpm dosed. <sup>b</sup> The cumulative cpm in each collection period was determined by summing the radioactivity of the metabolites present in the sample, summing the collection periods, and then dividing this sum by the total cpm dosed. <sup>c</sup> The cumulative amount of material in each collection period was determined by summing the amounts of the metabolites present in each sample, summing the collection periods, and then dividing this sum by the total amount of compound dosed.

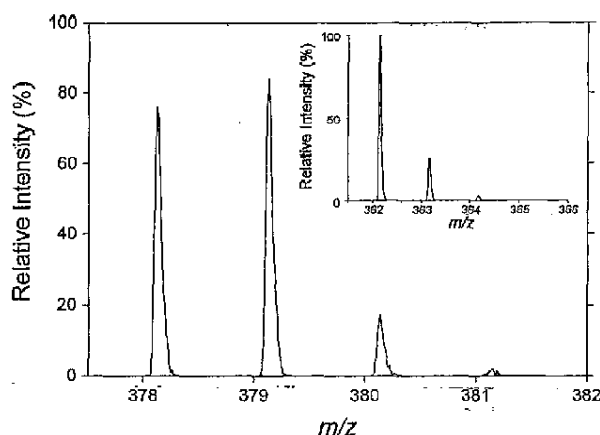


**Figure 6.** Nitrogen content derived from accurate mass measurement using a quadrupole-TOF-MS and a priori knowledge. Mass spectrometer was externally calibrated prior to LC/MS analysis. For predicting elemental composition of the monoisotopic molecular weight in the Analyst QS software (ABI SCIEX), the following limits were used on the basis of the assumption that this metabolite contained the diphenylpyrimidine core of the parent compound: maximum number of nitrogen and chlorine atoms were 8 and 2, respectively; minimum number of double bonds and rings combined was 8; and charge state was even.

## CONCLUSIONS

In this study, we elucidated the structure of each metabolite by MS<sup>\*</sup> and used this information to determine the number of nitrogen atoms in each analyte for quantification by CLND. This nitrogen information can also be achieved by determining the elemental composition of the structure. Elemental composition can be obtained using any of the currently available accurate-mass mass spectrometers. Since metabolites are derivatives of a known structure, that is, there is a priori knowledge, the mass accuracy of a time-of-flight (TOF)-MS is sufficient to deduce their elemental composition.<sup>9</sup> To illustrate this point, a bile sample was analyzed using a SCIEX Q-STAR quadrupole-TOF-MS following RP-HPLC. The spectrum of the 378 MH<sup>+</sup> metabolite is shown in Figure 6. On the basis of the intensity of the peak at 380.05  $m/z$  relative to the monoisotopic peak, this metabolite contained the two chlorine atoms from the parent compound. In addition, the monoisotopic (MH<sup>+</sup>) mass was an even number, indicating that the number of nitrogen atoms in the structure was odd. This and other information related to the parent structure were used to limit the possibilities for the proposed elemental composition (see figure legend). Within a 20-ppm error, only three possibilities were viable. The correct one, which was 12 ppm different from the calculated mass, was easily deduced from the parent structure.

We have demonstrated that LC/MS-CLND is a useful method for identification and quantification of metabolites in complex biological matrixes and for measuring material balance in vivo. The only requirement for quantification by CLND is the presence of nitrogen in the parent compound and the metabolites. Although it is possible to identify metabolites in complex matrixes with the proper controls, conclusive identification by MS is significantly easier if the parent also contains an atom or atoms in the core



**Figure 7.** Distinctive isotopic pattern identifies an in vivo metabolite of a  $^{13}C$  labeled compound. For comparison, the inset shows the isotopic pattern of the unlabeled parent compound, which is typical of a small molecule in this molecular weight range.

that provide a distinctive isotope signature. The compound used in this study contained two chlorine atoms that provided the signature. With compounds that do not contain halogens, we obtain distinctive isotopic signatures by synthesizing them with the stable isotopes  $^{13}C$  or  $^{18}O$  (Figure 7). Other stable isotopes that can be used are  $^2H$  and  $^{15}N$ . In addition, we use the specific isotopic pattern of the labeled compound to set the parameters for data-dependent MS experiments that automatically detect, record, and fragment only the compound and its metabolites. Using the methodology described in this report, we have been able to study the in vivo behavior of new analogues at a rate that kept pace with the synthesis of these compounds. For example, by studying the analogue series derived from CHIR 99021, we identified that particular chemical properties required for the activity of this series were also responsible for its in vivo metabolic liability.

Radioactivity is still the gold standard for in vivo ADME studies, but the ability to perform these same studies with LC/MS-CLND instead of radiolabeling provides a significant advantage to drug discovery and early development. Using this methodology, in vitro ADME assays can be rapidly validated so that they are used with confidence and in vivo structure-activity relationships related to distribution, metabolism, and excretion can be explored and identified. Thus, leading drug candidates can be optimized in a reasonable time frame and at a practical cost.

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